Kidney International, Vol. 57 (2000), pp. 332–338

A specific method for measurement of nitric oxide synthase enzymatic activity in peritoneal biopsies

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A specific method for measurement of nitric oxide synthase enzymatic activity in peritoneal biopsies.

Background. Nitric oxide (NO) is synthesized by NO synthase (NOS) isoforms that are expressed in the peritoneum. Thus far, NOS activity in the peritoneum has been assessed by nonspecific methods. We describe the application of a specific method for determination of NOS activity in rat and human peritoneal biopsies.

Methods. The L-citrulline assay is based on the stoechiometric production of NO and L-[^{3}H]-citrulline from L-[^{3}H]-arginine by NOS. The assay is technically difficult when applied on small samples with relatively low levels of NOS activity, which required specific procedures for extraction and samples processing. Reaction parameters ensuring assay linearity in the peritoneum were defined. Peritoneum lysates were also used for immunoblot analysis to identify the NOS isoforms involved.

Results. A significant NOS activity is detected in the normal peritoneum because of both Ca^{2+}-dependent and Ca^{2+}-independent NOS. The specificity of NOS activity has been demonstrated by various controls, including the NOS inhibitor L-NMMA. Competition experiments with L-valine and amino acid analyses have reasonably excluded the interference of endogenous arginase and L-arginine, which both might underestimate NOS activity. The procedure is sensitive; it detects a high range of NOS activities as well as the appropriate NOS isoforms in various tissues and conditions, as shown by correlations with immunoblot studies.

Conclusions. We have adapted and characterized the L-citrulline assay to measure specific NOS activities within the peritoneum. The peritoneum lysate assayed for NOS activity can also be used for characterizing NOS isoform expression by immunoblot analysis.

Nitric oxide (NO) is synthesized from L-arginine by a family of three NO synthase (NOS) isoforms that are expressed in a large variety of tissues and cells [1, 2]. The neuroneal NOS (nNOS, or NOS1) and the endothelial NOS (eNOS, or NOS3) are constitutively expressed in cells in which their activities are controlled by intracellular Ca^{2+} levels. In contrast, the inducible NOS (iNOS, or NOS2) remains quiescent until its transcription is activated by mediators such as lipopolysaccharides and/or cytokines [1]. Although iNOS activity is Ca^{2+} independent in native conditions, Venema et al have shown that recombinant iNOS can be activated by exogenous Ca^{2+} [3]. In addition to its involvement in neurotransmission and defense against infection, NO controls systemic vasodilation and affects microvascular permeability [2, 4]. Thus, it is not surprising that NO is considered as a potential regulator of peritoneal permeability, particularly in situations (long-term peritoneal dialysis or acute peritonitis) characterized by ultrafiltration failure [5–8]. Furthermore, recent studies have shown that NOS isoforms are expressed with variable intensity in human and rat peritoneum and that eNOS is the prevailing isoform in the peritoneum vasculature [9, 10].

To date, the NOS activity in the peritoneum has been assessed by measuring plasma or dialysate concentrations of nitrite and nitrate [6–8, 11, 12], the nitrite:L-arginine ratio [12], or cGMP [7, 11]. However, these three methods are limited because: (*a*) they detect by-products of NO and therefore lack specificity; (*b*) they do not allow discrimination between Ca^{2+}-dependent and Ca^{2+}-independent NOS activities; and (*c*) they could reflect systemic, rather than local, activation of NOS isoforms. The lack of direct demonstration of endogenous NOS activity at the tissue level has even raised the question of whether it is present in the normal peritoneum [6–8].

The biochemical transformation of L-arginine by NOS isoforms results in the stoechiometric production of NO and L-citrulline. That chemical reaction is the basis of the L-citrulline assay, a simple, sensitive, and specific method that is now the most recognized assay for NOS enzymatic activity [13, 14]. This technique is based on the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline, with control

Key words: nitric oxide, peritoneum, L-arginine, L-citrulline assay, infection, neurotransmission, dialysis membrane permeability.

Received for publication January 4, 1999
and in revised form June 14, 1999
Accepted for publication August 20, 1999

reactions (blanks) performed either (*a*) with a specific inhibitor of NOS, (*b*) in absence of the cofactor nicotinamide adenine dinucleotide phosphate (NADPH), or (*c*) with an extract that has been boiled prior to the incubation. Because the reaction can be performed with or without Ca^{2+}, it can discriminate between Ca^{2+}-dependent and Ca^{2+}-independent NOS activities. The L-citrulline assay has been widely used to demonstrate NOS activity in a variety of cells and tissues [13, 14]. However, NOS activity is relatively unstable, and the L-citrulline assay is technically difficult when applied on small, biopsy samples or on tissues with relatively low levels of NOS activity [13, 14].

In this study, we have adapted and characterized the L-citrulline assay in order to measure the specific Ca^{2+}-dependent and Ca^{2+}-independent NOS activities within the peritoneum. We show that the peritoneum lysate assayed for NOS activity can also be used for studying the expression of the NOS isoforms by immunoblot analysis.

METHODS

Tissue samples

Samples of rat visceral peritoneum were taken from eight-week-old male Sprague-Dawley rats ($N = 5, 307 \pm 6$ g body wt) with access to standard rat chow. Preliminary studies for arginase and nonspecific NOS activities were conducted in 12-week-old rats ($N = 4$, body weight: 397 ± 18 g). The animals were anesthetized with 90 mg/kg subcutaneous (SC) sodium pentobarbital (Nembutal, Ceva, Brussels, Belgium) and sacrificed by exsanguination. The abdomen was opened by a midline incision for tissue sampling. Some samples were similarly obtained in rats with peritonitis (intraperitoneal infection for 7 days). Samples from dog heart ventricle ($N = 6$, obtained just after sacrifice) were used as a positive control for NOS activity [15]. Paraumbilical biopsy samples of human parietal peritoneum were obtained from a series of six normal subjects (34 ± 12 years old, 67% males) at the time of nephrectomy (living donors, $N = 2$) or laparotomy for benign surgery ($N = 4$). Other human tissues, including kidney and brain, were obtained at autopsy. The animal experiments were conducted in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The use of the human samples was approved by the University of Louvain Ethical Review Board.

Tissue extraction and processing

After washing in ice-cold phosphate-buffered saline, peritoneal samples were divided in two parts. A small part of the sample was used for fixation in 4% paraformaldehyde, paraffin embedding, and routine pathological examination, whereas the major part of the sample was snap frozen in liquid nitrogen and processed for protein extraction. The frozen samples were finely grounded in a specific mortar containing liquid nitrogen (Resco Trade, Kortrijk, Belgium) and suspended in (2 mL/g of tissue) ice-cold buffer [50 mmol/L Tris, pH 7.4, containing 0.1 mmol/L egtazic acid (EGTA), 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 2 mmol/L β-mercaptoethanol, 5 μmol/L leupeptin, and 4 μmol/L pepstatin]. The suspension was homogenized in three passes of 10 seconds at 22,000 r.p.m. with Ultra-Turrax® (Labortechnik, Staufen, Germany) and then sonicated with 10 brief pulses at 40% intensity (Branson Sonifier B-12, Danbury, CT, USA). The resulting homogenate was centrifuged at $6000 \times g$ (Sigma 113 centrifuge, Osterode am Harz, Germany) for 10 minutes at 4°C. The postnuclear supernatant (total protein extract) was kept on ice for determination of protein concentrations with the Bradford method (Bio-Rad, Melville, NY, USA) and for amino acid analysis with ion-exchange chromatography (Biochrom 20 Analyzer; Pharmacia Biotech, Rosendaal, The Netherlands). All samples were aliquoted and stored at −80°C.

Measurement of nitric oxide synthase enzymatic activity

Nitric oxide synthase activity was measured by the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline [13]. One volume of tissue extract containing 300 to 400 μg of total protein and 20 mmol/L CHAPS was added to 200 μL of reaction mixture [50 mmol/L Tris buffer, pH 7.4, containing 10 mmol/L dithiothreitol (DTT), 10 μmol/L THB_4, 10 μg/mL calmodulin, 1 mmol/L NADPH, 4 μmol/L flavin adenine dinucleotide (FAD), 4 μmol/L flavin mononucleotide (FMN), 2 μmol/L L-arginine, and 10^{-3} mCi/mL L-[^{3}H]-arginine]. The assay was carried out at 37°C [15], and it was terminated with 2 mL of ice-cold stop buffer (20 mmol/L CH_3COONa, pH 5.5, containing 2 mmol/L EDTA, 0.2 mmol/L EGTA, and 1 mmol/L L-citrulline). As L-[^{3}H]-citrulline is neutral at pH 5.5, it was separated from the incubation mixture by cation exchange resin (Dowex AG 50 W-X8; Bio-Rad) to which L-[^{3}H]-arginine adhered. After elution with 2 mL of water, L-[^{3}H]-citrulline was quantitated by 16 mL liquid scintillation counting (SL 3000; Intertechnique, Plaisir, France). Background radioactivity (in percentage of total radioactivity) was measured in order to assess the quality of the L-[^{3}H]-arginine stock (background radioactivity below 3%). The possibility of arginase activity in the peritoneum extracts was studied by measuring the formation of L-[^{3}H]-citrulline in samples without (control conditions) or with L-valine (concentrations from 1 μmol/L to 1 mmol/L). These conditions were controlled for experimental pH [14, 16].

The specific NOS activity (in pmol citrulline produced/mg protein/min) was determined by subtracting counts obtained in the presence of 1 mmol/L L-NMMA, a spe-

cific inhibitor of NOS, from counts obtained in the absence of L-NMMA. Other controls for nonspecific NOS activity, including incubations without NADPH and incubations with a previously boiled extract, were performed in preliminary experiments on four control rats as well as in some subsequent experiments [13, 14].

Assays were systematically performed with Ca^{2+} (1 mmol/L $CaCl_2$) or without Ca^{2+} (0 mmol/L $CaCl_2$, 2 mmol/L EGTA, and 2 mmol/L EDTA) to measure total versus Ca^{2+}-independent NOS activities [13]. The Ca^{2+}-dependent NOS activity was calculated as total NOS activity minus Ca^{2+}-independent NOS activity. All assays were performed in duplicate on aliquoted samples (to avoid freezing/thawing cycles). The results were normalized for protein content.

Determination of linear parts of L-[^{3}H]-citrulline formation curves as a function of total protein concentration and incubation time. The formation of L-[^{3}H]-citrulline in rat peritoneum extracts was characterized as a function of the total protein concentration (from 50 to 800 μg of total protein in 200 μL of reaction mixture) for a 30-minute incubation at 37°C and as a function of the incubation time (from 10 to 120 min) at 37°C with a protein concentration of 400 μg in order to determine the linear parts of the curves [13]. The latter experiment was performed with an extract from rat with peritonitis in order to determine the linearity patterns of both total and Ca^{2+}-independent NOS activities [10].

Immunoblot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed to detect NOS isoforms [9] in the lysate used for L-citrulline assay, with monoclonal antibodies raised against human eNOS and nNOS, and mouse iNOS (Transduction Laboratories, Lexington, KY, USA), and a polyclonal antiserum against mouse iNOS (Biomol, Plymouth Meeting, PA, USA). Briefly, the extracts were solubilized by heating at 95°C for two minutes in sample buffer [1.5% SDS, 10 mmol/L Tris-HCl, pH 6.8, 0.6% DTT, and 6% (vol/vol) glycerol]. Proteins (40 to 120 μg/lane) were separated by electrophoresis through 0.1 × 9 × 6 cm 7.5% acrylamide slabs and were transferred to nitrocellulose. The membranes were briefly stained with Ponceau Red to check the efficiency of transfer. Destained membranes were blocked for 30 minutes at room temperature in blotting buffer comprising 5% nonfat dry milk, 50 mmol/L $NaPO_4$, 150 mmol/L NaCl, 0.05% Tween 20, pH 7.4, followed by incubation with the anti-NOS antibody diluted in 2% bovine serum albumin, 50 mmol/L $NaPO_4$, 150 mmol/L NaCl, 0.05% Tween 20, pH 7.4, at 4°C for 16 to 18 hours. The membranes were then washed and incubated for one hour at room temperature with a peroxidase-labeled antimouse or antirabbit antibody (1:5000 dilution; Dako, Glostrup, Denmark). After washing, immunoblots were visualized after one minute of enhanced chemiluminescence (Amersham, Little Chalfont, UK).

Reagents and supplies

L-[^{3}H]-arginine was from Amersham and liquid scintillation from Lumac (Groningen, The Netherlands). Other reagents and supplies were from Boehringer (Mannheim, Germany) and Sigma Chemical (St. Louis, MO, USA).

Data analysis

Data are presented as mean ± SEM. Comparisons between the results from different groups were performed using Student's *t*-test or one-way analysis of variance, as appropriate. Nonlinear regressions were made with the software Prism® (GraphPad, San Diego, CA, USA).

RESULTS

Protein extraction and morphological examination of peritoneal biopsies

Protein extraction yielded 25 ± 7 μg protein/mg tissue in rat (N = 5, tissue wt, 532 ± 82 mg) and 49 ± 8 μg protein/mg tissue in human (N = 6, tissue wt, 404 ± 92 mg) index samples. Pathological examination confirmed the nature of the tissue, its normal architecture, and the lack of overt peritonitis in the normal samples, as well as typical signs of peritonitis (edema, infiltrate) in infected rats (data not shown).

Specificity of nitric oxide synthase activity in the peritoneum

Arginase is a major L-arginine–consuming enzyme that might interfere with the NOS assay in the peritoneum [17]. The possibility of arginase activity in the noninfected rat peritoneum was studied by measuring the formation of L-[^{3}H]-citrulline in control conditions (absence of L-valine) and with increasing concentrations of L-valine (an inhibitor of arginase) in the extract [14, 16]. As shown in Figure 1A, the addition of L-valine to the extract had no significant influence on the production of L-citrulline. This was true for L-valine concentrations ranging from 1 μmol/L to 1 mmol/L (higher concentrations were associated with aspecific interactions with the resin). Similarly, the presence of endogenous L-arginine in the extracts might also interfere with the assay. Amino acid analysis showed that the levels of endogenous L-arginine in the extracts used for the NOS assay were below the detection limit of 1 μmol/L in the human and rat samples tested (N = 6).

To determine total versus nonspecific NOS activity in the peritoneum (Fig. 1B), the L-citrulline assay was performed in rat peritoneum samples (*a*) with L-NMMA, (*b*) in absence of NADPH, or (*c*) after boiling the extract.

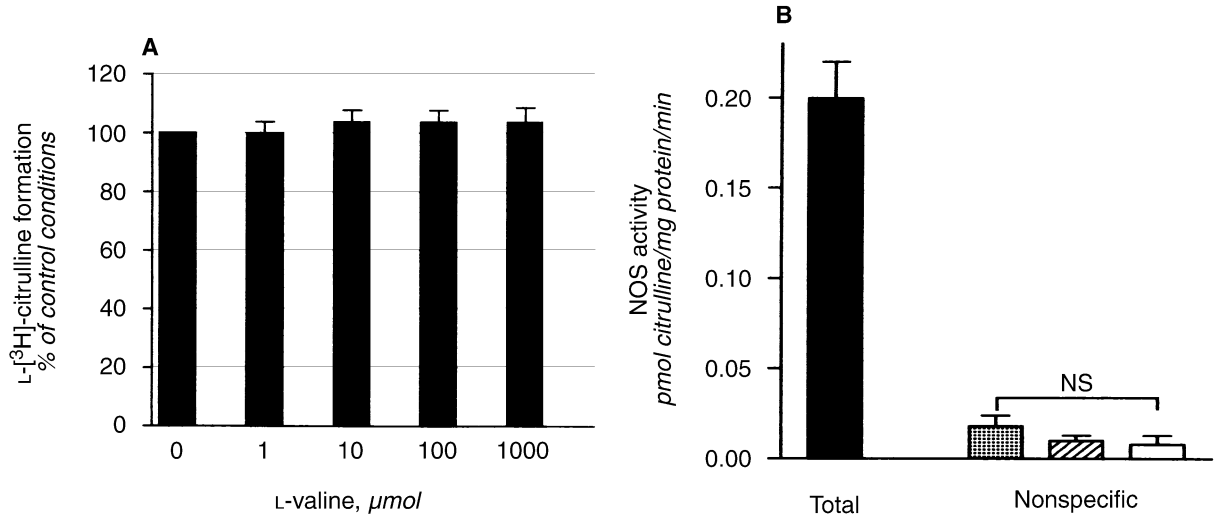

Fig. 1. Specificity of nitric oxide synthase (NOS) activity in the peritoneum. (*A*) Analysis of arginase activity: Effect of L-valine on the formation of L-citrulline. To detect a potential interference between arginase and NOS in the peritoneum, the formation of L-citrulline (measured by the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline) was studied in control conditions (absence of L-valine) and with increasing concentrations of L-valine (from 1 μmol/L to 1 mmol/L) in peritoneal extracts from noninfected rats. When compared with control conditions (ΔCPM, 1153 ± 15 or 100%), the addition of L-valine to the extract has no significant effect on the production of L-citrulline (maximum increase, 4 ± 4%; ANOVA, P = NS). Determinations were performed in duplicate for three different samples. (*B*) Determination of total versus nonspecific NOS activity. Total NOS activity (■; in pmol citrulline produced/mg protein/min) was measured by the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline in rat visceral peritoneum. Nonspecific NOS activity was determined by three different controls: (▩) addition of the NOS inhibitor L-NMMA to the extract, (▨) removal of the cofactor NADPH from the reaction mixture, and (□) boiling the extract prior to incubation. The nonspecific activities obtained with the three controls are similar and represent 4 to 9% of the total NOS activity. Determinations were performed in duplicate in four different samples.

Nonspecific NOS activities (in pmol citrulline/mg protein/min, percentage of total NOS) obtained with the three types of controls (with L-NMMA, 0.018 ± 0.006, 9%; without NADPH, 0.01 ± 0.003, 5%; boiled extracts, 0.008 ± 0.005, 4%) were similar. The mean background radioactivity for these experiments was 2.25%.

Determination of nitric oxide synthase activities in human and rat peritoneum

The relationships between L-[^{3}H]-citrulline formation, incubation time and total protein concentration were examined in the rat peritoneum (Fig. 2A) to define the conditions ensuring assay linearity. An incubation time of 30 minutes at 37°C corresponded to the linear part of the curve of L-[^{3}H]-citrulline formation with (total NOS) and without (Ca^{2+}-independent NOS) Ca^{2+} (left panel). Similarly, a total protein concentration of 300 to 400 μg corresponded to the linear part of the assay (right panel). These conditions were subsequently used to measure NOS enzymatic activities in all samples.

A significant level of specific NOS activity was detected in rat visceral peritoneum and human parietal peritoneum (Fig. 2B). This activity was comparable with that detected in dog heart, the tissue used in our study as a positive control [15, 18]. Specific, total NOS activities reached 0.051 ± 0.012 pmol citrulline/mg protein/min in rat peritoneum and 0.033 ± 0.008 pmol citrulline/mg protein/min in human peritoneum, as compared with 0.045 ± 0.003 pmol citrulline/mg protein/min in dog heart. The NOS activity in the peritoneum was attributed to both Ca^{2+}-dependent and Ca^{2+}-independent NOS isoforms. The Ca^{2+}-dependent NOS activity was 0.021 ± 0.008 and 0.016 ± 0.005 pmol citrulline/mg protein/min in rat and human peritoneum, respectively. The Ca^{2+}-independent NOS activity was 0.03 ± 0.006 and 0.017 ± 0.006 pmol citrulline/mg protein/min in rat and human peritoneum, respectively. The background radioactivity for these experiments averaged 2.7%.

The intra-assay variability in peritoneal samples was 2 ± 0.4% for total NOS and 3 ± 0.7% for Ca^{2+}-independent NOS activities (N = 11). The interassay variability was 7 ± 2% for total NOS (N = 7) and 8 ± 1% for Ca^{2+}-independent NOS (N = 5) activities. It must be noticed that NOS activity was age dependent in rats and was affected by the freezing/thawing cycles of the samples.

The specific NOS activity was also determined in other human tissues, including kidney and brain, characterized by their high endogenous activity [2], as well as in the peritoneum of an infected rat (Table 1). These experiments demonstrated the assay's ability to detect high NOS activities and to differentiate between elevated Ca^{2+}-dependent (brain and kidney samples) and Ca^{2+}-independent (infected peritoneum) NOS activities.

Immunoblot analysis of nitric oxide synthase isoforms

The extracts used for the L-citrulline assay were submitted to SDS-PAGE and immunoblotting in order to

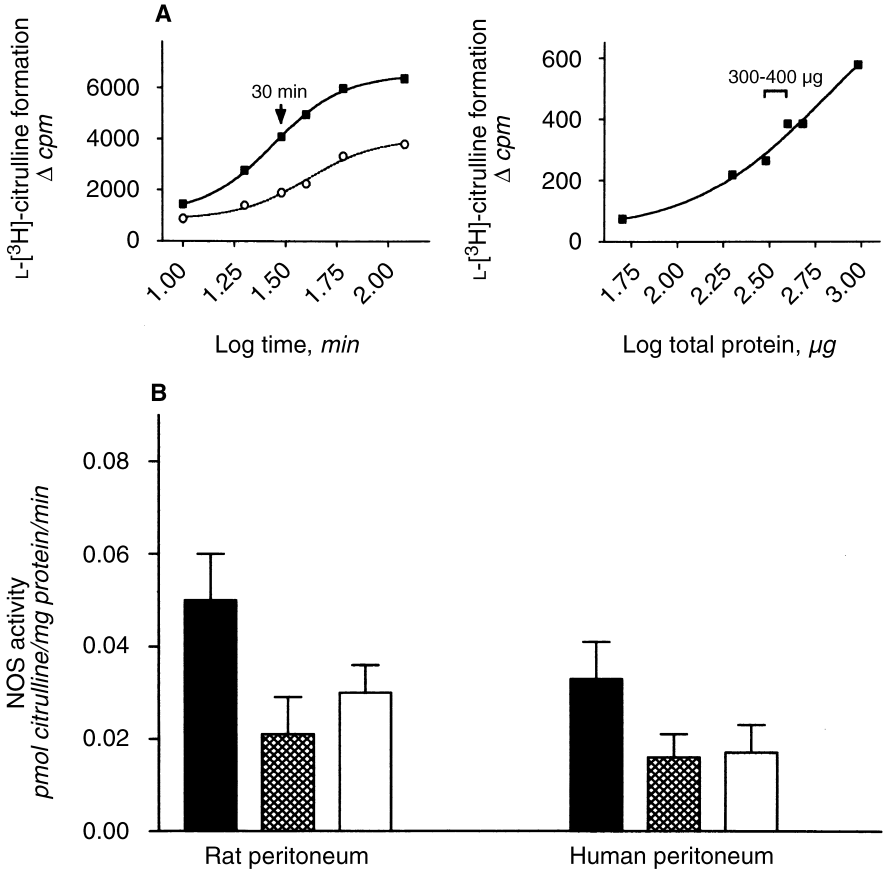

Fig. 2. L-citrulline assay for specific, Ca^{2+}-dependent and Ca^{2+}-independent nitric oxide synthase (NOS) activities in rat and human peritoneum. (*A*) L-[^{3}H]-citrulline formation curves as a function of incubation time (left panel) and total protein concentration (right panel). Symbols are: (■) total NOS; (○) Ca^{2+}-independent NOS. The standard conditions of the assay (incubation time, 30 min; protein concentration, 300 to 400 µg) are in the linear parts of the curves. Each determination was done in duplicate in a sample of rat peritoneum. Note that a rat with peritonitis was used to perform the time course analysis in order to get an increased total and Ca^{2+}-dependent NOS. (*B*) Specific, total NOS activity (in pmol citrulline produced/mg protein/min, ■) was measured by the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline in rat visceral peritoneum ($N = 5$) and in human parietal peritoneum ($N = 6$). The assays were performed in the presence or in the absence of Ca^{2+} in order to measure Ca^{2+}-dependent (⊠) and Ca^{2+}-independent (□) NOS activities. The values shown are those obtained after subtraction of nonspecific NOS (L-NMMA controls). Each determination was done in duplicate for all samples. Paired *t*-tests showed no statistically significant differences between Ca^{2+}-dependent and Ca^{2+}-independent NOS activities.

Table 1. Nitric oxide synthase (NOS) activities in human kidney, human brain, and rat infected peritoneum

	NOS activity		pmol citrulline/mg protein/min
	Total	Ca^{2+}-dependent	Ca^{2+}-independent
Human kidney cortex	0.3780 ± 0.0118	0.3579 ± 0.0114	0.0201 ± 0.0045
Human brain cortex	0.9345 ± 0.0283	0.9081 ± 0.0580	0.0263 ± 0.0155
Rat infected peritoneum	0.5246 ± 0.0031	0.3616 ± 0.0095	0.1631 ± 0.0065

Data are means ± SEM. The assay was performed in quadruplicate from one sample.

demonstrate the selective expression of the three NOS isoforms in these samples (Fig. 3A). Each monoclonal antibody recognized the appropriate NOS isoform, as shown by detection of a major band in control extracts prepared from pituitary tissue (nNOS, 155 kd), endothelial cells (eNOS, 140 kd), or macrophages (iNOS, 130 kd). It must be noticed that the monoclonal anti-iNOS cross-reacted with an upper band corresponding to eNOS (compare the kidney and peritoneum samples). Each of the two Ca^{2+}-dependent NOS isoforms was highly expressed either in brain (nNOS) or in kidney and peritoneum (eNOS), whereas iNOS was found to be expressed with variable intensity in infected peritoneum and brain. Despite a lack of specificity of the anti-iNOS, the expression of iNOS in human brain (autopsy samples) was confirmed by the resolution of the gels (allowing to discriminate the specific mass of the NOS isoforms) [10] and the use of other anti-iNOS antibodies (data not shown).

Subsequent analyses demonstrated that detection of iNOS in the rat peritoneum by immunoblotting was related to the white blood cell count in the peritoneal rinsing solution, a value probably reflecting the amount of macrophages in the tissue (Fig. 3B). The intensity of the signal for iNOS was very weak or even undetected in some noninfected samples. Similar findings were obtained with a polyclonal antiserum against iNOS (Fig. 3C).

DISCUSSION

In this study, we describe the adaptation of the L-citrulline assay to measure NOS activities in human and rat peritoneum samples. The specificity of the assay has been demonstrated by three types of controls, including the NOS inhibitor L-NMMA. Competition experiments with L-valine and amino acid analyses have reasonably excluded the potential interference of endogenous arginase

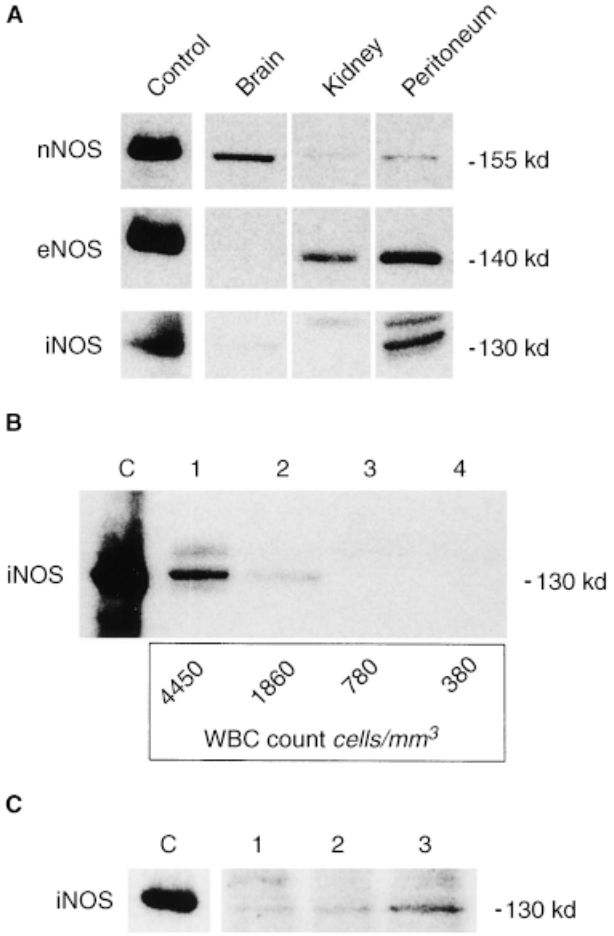

Fig. 3. Immunoblot analysis of nitric oxide synthase (NOS) isoforms in tissue lysates. (*A*) Two micrograms of positive control (nNOS, rat pituitary; eNOS, bovine aortic endothelial cells; iNOS, mouse macrophages) as well as lysates from human brain, human kidney, and infected rat peritoneum (40 μg of total protein per lane) were submitted to SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody against nNOS, eNOS, or iNOS (dilution 1:2500). Each antibody recognizes the appropriate isoform expressed in the control, as shown by the major band at 155 kd (nNOS), 140 kd (eNOS), and 130 kd (iNOS). The lysates used for the L-citrulline assay contain the three NOS isoforms, expressed according to the tissue of origin: nNOS is mostly expressed in the brain, eNOS in the kidney and peritoneum, and iNOS (with variable intensity) in the brain and infected peritoneum. Note that the monoclonal anti-iNOS cross-reacts with an upper band corresponding to eNOS in kidney and peritoneum samples. The film was exposed for two minutes. (*B*) The detection of iNOS (130 kd) in the peritoneum correlates with the amount of white blood cells (WBCs) in peritoneal rinsing solution. Peritoneum samples obtained from rats with overt (lane 1), mild (lane 2), or apparently absent (lanes 3 and 4) peritonitis were submitted to immunoblot analysis with the monoclonal anti-iNOS antibody (dilution 1:2500). The intensity of the signal corresponding to iNOS is correlated with the WBC count, taken as an index of the macrophage content of the peritoneum (a 15 mL saline solution was used to rinse the peritoneum before sampling). Forty micrograms (lane 1) or 120 μg (lanes 2 through 4) of total protein were loaded. Lane C is the positive control for iNOS (mouse macrophages lysate). The film was exposed for seven minutes. (*C*) Detection of iNOS (130 kd) in noninfected (lanes 1 and 2) and infected (lane 3) rat peritoneum (100 μg total protein per lane), using a polyclonal antiserum against iNOS (dilution 1:2000). Lane C is mouse macrophage lysate. The film was exposed for seven minutes.

and L-arginine, which both might underestimate NOS activity in the samples. The assay is sensitive; it detects a high range of NOS activities as well as the appropriate NOS isoforms in a variety of tissues and conditions. Finally, reproducibility is attested by a small intra-assay and interassay variability. Thus far, the production of NO in the peritoneum has been evaluated by measuring plasma or dialysate levels of nitrate and nitrite, L-arginine, or cGMP [6–8, 11, 12]. These methods lack specificity, do not allow discrimination between NOS isoforms, and might reflect systemic, rather than local, activation of NOS activity. The latter point explains why local production of NO in the normal peritoneum remains questioned [6–8].

The L-citrulline assay is now the usual assay for NOS activity for several reasons [13, 14]. First, the assay is relatively simple and highly reproducible, with a very high sensitivity. Second, the availability of specific inhibitors of NOS ensures the specificity of the assay. It is true that citrulline can be formed via an alternate pathway (the urea cycle), but the latter process is restricted to liver and kidney, because most cells do not express the enzymes catalyzing the formation of citrulline from ornithine [19]. Third, this method permits measurement of specific NOS activity, as well as Ca^{2+}-dependent and Ca^{2+}-independent NOS activities. However, NOS activity is labile and the assay is difficult to perform when used on small samples (such as biopsies) or on tissues or cells expressing low levels of NOS [13, 14].

Several technical modifications allowed us to apply the L-citrulline assay and to improve the yield of information obtained from one single peritoneal biopsy. First, the homogenization procedure was optimized to give a high yield of protein extraction, which allowed us to process small peritoneal samples, including human biopsies. Second, we had to select experimental conditions ensuring assay linearity in the peritoneum. As shown in Figure 2, the conditions routinely used for the reaction were in the linear parts of the L-[^{3}H]-citrulline formation curves. Third, the processing of the samples (frozen immediately after dissection, kept in liquid nitrogen during the initial steps of homogenization, and aliquoted to avoid freezing/thawing cycles) allowed a better preservation of NOS activity than other methods. Fourth, the same postnuclear supernatant obtained with our extraction protocol was used for the L-citrulline assay or for immunoblot analysis, as shown by the identification of the three NOS isoforms (Fig. 3). The latter point is particularly important when considering that NOS isoforms have distinct intracellular distribution [2, 10], as they might escape detection by immunoblot if the analysis is performed on a subcellular fraction. Thus, the great advantage—a decisive gain for correlation studies—of the procedure detailed in our study is that a single peritoneum biopsy can be used for various experimental approaches that

include morphological examination, immunoblot analyses, and the L-citrulline assay.

This study demonstrates a specific NOS activity in the normal human and rat peritoneum. When compared with other tissues tested with the same methodology (Table 1), the level of NOS activity within the peritoneum is relatively low but is similar to that measured in the heart left ventricle, which was used here as a positive control [15, 18]. The NOS activity in the peritoneum is due to both Ca^{2+}-dependent and Ca^{2+}-independent NOS isoforms. By analogy with the normal respiratory epithelium, the presence of inducible, Ca^{2+}-independent NOS in the normal peritoneum might reflect a chronic local activation of the rat immune system [1]. Immunoblot analysis (Fig. 3A) showed that the three NOS isoforms can be identified with variable intensity in the peritoneum, as previously reported [10]. However, it must be noticed that iNOS detection on immunoblot appears to reflect the macrophage content of the peritoneum (Fig. 3 B, C). In that respect, the NOS assay might be more sensitive than immunoblotting to detect the (Ca^{2+}-independent) activity corresponding to iNOS.

Table 1 shows that the assay detects different types of activities according to the tissue of origin. Within the peritoneum, infection is associated with increased Ca^{2+}-independent NOS, as expected, but also with increased levels of Ca^{2+}-dependent NOS, probably related to capillary proliferation and increased eNOS expression [10]. In the case of peritonitis, the macrophage infiltrate might also be associated with increased arginase activity and subsequent underestimation of NOS activity [17].

Nitric oxide is thought to play an important role in regulating the permeability and the ultrafiltration capability of the peritoneal membrane [5, 7, 9, 10]. This role of NO might involve regulation of vascular tone and permeability [1, 4], two parameters that control the rate of dissipation of the osmotic gradient across the peritoneal membrane [5]. Alternatively, NO might alter the quality of the peritoneum via a toxicity mediated by reactive nitrogen species [20]. We believe that the use of the L-citrulline assay in human or rat peritoneal biopsies will be useful for further studies focusing on the role played by NO in the normal or diseased peritoneum.

ACKNOWLEDGMENTS

These studies were supported in part by the Fonds National de la Recherche Scientifique (crédit 9.4540.96) and grants from Baxter. Part of these results was presented at the 31st Annual Meeting of the American Society of Nephrology and was published in abstract form (*J Am Soc Nephrol* 9:191 A, 1998). We thank Mrs. A. Piech for assistance; Profs. J-P. Squifflet, Ch. Sindic, and R. Beauwens for help in providing tissue samples; Prof. M-F. Vincent for amino acid analyses; and Prof. C. van Ypersele for his continuous support.

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